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(54) Title: INHIBITION OF TNF ACTIVITY (57) Abstract The bioactivity of TNF is inhibited by administering heparin or a derivative thereof along with a soluble TNF receptor. The heparin or derivative thereof can be administered simultaneously with the soluble TNF receptor, either in separate compositions or in compositions containing both heparin or a derivative thereof and at least one soluble TNF receptor. The heparin or derivative may also be administered without the soluble TNF receptor and still effect some amount of inhibition of TNF bioactivity.		

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INHIBITION OF TNF ACTIVITY

Field of the Invention

5 The present invention is directed to a method and pharmaceutical compositions for inhibiting activity of tumor necrosis factor (TNF).

Background of the Invention

10 Tumor necrosis factor (TNF) is a pro-inflammatory cytokine produced by a wide spectrum of cells. It has a key role in defending the host, mediating complex cellular responses of different, and even contrasting, nature (Aggarwal et al, 1996). In excess, TNF may have detrimental systemic effects. Two specific high affinity cell surface receptors, the p55 TNF-receptor (p55 TNF-R) and the p75 TNF-receptor (p75 TNF-R), function as transducing elements, providing the
15 intracellular signal for cell responses to TNF. The extracellular parts of the TNF-Rs, known as soluble TNF-Rs, were formerly referred to as TBP-I and TBP-II respectively (see Wallach, US patent 5,359,037 and Tartaglia et al., 1992; Loetscher et al., 1991).

 The biological effects of TNF depend upon its concentration and site of production. At low concentrations, TNF may produce desirable homeostatic and
20 defense functions. For example, these effects may destroy tumor cells or virus infected cells and augment antibacterial activities of granulocytes. In this way, TNF contributes to the defense of the organism against infectious agents and to recovery from injury. However, at higher concentrations, systemically or in certain tissues, TNF can synergize with other cytokines, notably interleukin-1, to aggravate many
25 inflammatory responses. Additionally, the effects of TNF- α , primarily on the vasculature, are now known to be a major cause for symptoms of septic shock (Tracey et al, 1986). In some diseases, TNF may cause excessive loss of weight (cachexia) by suppressing activities of adipocytes and by causing anorexia.

 TNF has been found to induce the following activities (together with

interleukin-2): fever, slow-wave sleep, hemodynamic shock, increased production of acute phase protein, decreased production of albumin, activation of vascular endothelial cells, increased expression of major histocompatibility complex molecules, decreased lipoprotein lipase, decreased cytochrome P450, decreased plasma zinc and iron, fibroblast proliferation, increased synovial cell collagenase, increased cyclo-oxygenase activity, activation of T cells and B cells, and induction of secretion of the cytokines, TNF itself, interleukin-1 and interleukin-6.

Because of its pleiotropic effects, TNF has been implicated in a variety of pathologic states in many different organs of the body. In blood vessels, TNF promotes hemorrhagic shock, down-regulates endothelial cell thrombomodulin, and enhances a procoagulant activity. It causes adhesion of white blood cells, and probably of platelets, to the walls of blood vessels, and so may promote processes leading to atherosclerosis, as well as to vasculitis.

TNF activates blood cells and causes the adhesion of neutrophils, eosinophils, monocytes/macrophages and T and B lymphocytes. By inducing interleukin-6 and interleukin-8, TNF augments the chemotaxis of inflammatory cells and their penetration into tissues. Thus, TNF has a role in the tissue damage of autoimmune disease, allergies and graft rejection.

TNF has also been called cachectin because it modulates the metabolic activities of adipocytes and contributes to the wasting and cachexia accompanying cancer, chronic infections, chronic heart failure and chronic inflammation. TNF may also have a role in tissue damage of autoimmune diseases, allergies, and graft rejection.

TNF also has metabolic effects on skeletal and cardiac muscle. It also has marked effects on the liver: it depresses albumin and cytochrome P450 metabolism and increases production of fibrinogen, α -Acid Glycoprotein (AGP) and other acute phase proteins. It can also cause necrosis of the bowel.

In the central nervous system, TNF crosses the blood-brain barrier and induces fever, increased sleep and anorexia. Increased TNF concentration is also associated with multiple sclerosis. It also causes adrenal hemorrhage and affects production of steroid hormones, enhances collagenase and PGE-2 in the skin, and causes the breakdown of bone and cartilage by activating osteoclasts.

Thus, TNF is involved in the pathogenesis of many undesirable inflammatory conditions, in autoimmune disease, graft rejection, vasculitis and atherosclerosis. It appears to have a role in heart failure, in the response to cancer and in anorexia nervosa. For these reasons, means have been sought to inhibit the activity of TNF as a way to control a variety of diseases.

While exploring ways for antagonizing the destructive potential of TNF in certain clinical conditions, investigators looked for natural TNF inhibitors (Engelmann et al, 1989; Engelmann et al, 1990; Seckinger et al, 1989; Olsson et al, 1989). Such agents, first detected in urine, were structurally identical to the extracellular cytokine binding domains of the two membrane associated TNF-Rs (Nophar et al, 1990). These shed soluble TNF-Rs (sTNF-Rs) can compete for TNF with the cell surface receptors and thus block the cytokine activity.

However, interactions between the TNF-Rs and their ligand are much more complex than initially thought. At physiological concentrations, the trimeric and bioactive TNF molecules decay, dissociating into inactive monomeric forms (Petersen et al, 1989; Aderka et al, 1992). Addition of sTNF-Rs to the TNF trimers promotes formation of complexes between them, which can preserve and prevent the decay of the active, trimeric forms of TNF (Aderka et al, 1991; De Groote et al, 1993). This bioactive TNF may dissociate from this complex to replace free TNF which decayed, thus maintaining a constant concentration of free, bioactive, trimeric cytokine. This reversible interaction between the soluble receptors and their ligand expands the functions attributable to the TNF receptors. In their soluble form, the TNF-Rs may

serve as:

- (a) TNF antagonists (when present in large excess relative to TNF);
- (b) TNF carrier proteins (between body compartments);
- (c) slow release reservoirs for bioactive TNF;
- 5 (d) stabilizers of the TNF's bioactive form (which may also prolong the half-life of TNF); and
- (e) TNF "buffers", by inhibiting the effects of high TNF concentrations and presenting it at low and well-controlled levels to the cells (Aderka et al, 1992).

10 The functions of the TNF receptors, thus, are not limited to signal transduction but include, in their soluble forms, extracellular regulatory roles affecting local and systemic bioactive TNF availability.

TNF and Disease

Examination of patients with septic shock due to meningococemia
15 revealed that the ratio of TNF/sTNF-Rs was higher in patients with a fatal outcome compared to patients who recovered, suggesting a critical imbalance between the ligand and its inhibitors (Girardin et al, 1994). Neutralization of the excess TNF seemed to be the preferred next step.

Indeed, dimeric Fc fusion constructs of the p55 sTNF-R, but not of the
20 p75 sTNF-R, were found to protect mice from lethal doses of LPS (Evans et al, 1994) if administered not later than 1-3 hours post LPS (Peppel et al, 1991; Mohler et al, 1993; Ashkenazi et al, 1991; Lesslauer et al, 1991). This suggests that septic shock manifestations occur if the initial high TNF concentrations generated are not buffered by adequate soluble receptor concentrations during that narrow window of time.

25 To add to the growing confusion, neutralization of TNF with monoclonal anti-TNF Ab (Abraham et al, 1995; Kaul, et al, 1996) or p55 sTNF-R IgG1 (Leighton et al, 1996) in patients with severe sepsis or septic shock yielded

conflicting results. In one study, the antibodies proved ineffective (Abraham et al, 1995), while in the other, administration of the antibodies benefited only those patients with baseline interleukin-6 levels higher than 1000 pg/ml but increased the mortality of those with lower interleukin-6 levels (Kaul et al, 1995). In another randomized trial, septic patients given a recombinant dimer consisting of sTNF-R/Fc portion of IgG1 had higher mortality (48-53%) as compared to placebo-treated patients (30%) (Suffredini et al, 1994; Fisher et al, 1996). Interestingly, the higher the dose of the sTNF-Rs administered, the higher was the patient mortality (Fisher et al, 1996). It was suspected that the effective removal of circulating TNF may result in the exacerbation of the systemic infection (Fisher et al, 1996). In contrast, in a recent study the administration of similar soluble Fc receptor constructs apparently benefitted septic patients irrespective of their serum interleukin-6 concentrations, with a 36% mortality reduction compared to placebo treated individuals (Leighton et al, 1996). These contradictory data give the impression that the administration of sTNF-Rs may have a very narrow therapeutic index which would be difficult to individualize at bedside. Too much of the receptors may totally neutralize TNF, exacerbating the systemic infection, while too little of the receptors may not neutralize enough TNF, resulting in septic shock and the patient's demise. The real challenge is to fine-tune the sTNF-R dose in order to permit low TNF levels to exert their protective effects. Thus, paradoxically, lower doses of sTNF-Rs than previously employed (Fisher et al, 1996), rather than higher ones, may benefit septic shock patients.

Since the TNF neutralization should not be complete, but should be aimed to leave low amounts of bioactive TNF to exert the desired beneficial effects, natural soluble TNF receptors may be ideally suited for this purpose.

TNF is also a pivotal cytokine in the pathogenesis of Crohn's Disease, a chronic and disabling disorder of the bowel, and is, therefore, a prime target for specific immunotherapy (Braegger et al, 1992; MacDonald et al, 1990; Breese et al,

1994). Indeed, treatment of Crohn's Disease patients with chimeric anti-TNF monoclonal antibodies induced a spectacular remission in patients unresponsive to conventional therapy (van Dullemen et al, 1995). Whether slow release preparations of sTNF-Rs (Eliaz et al, 1966) will have identical effects on the course of this disease remains to be determined.

In another autoimmune disorder, rheumatoid arthritis, it was demonstrated that the serum sTNF-Rs may be useful in monitoring disease activity (Cope et al, 1992; Roux-Lombard et al, 1993). It was shown that despite the presence of high levels of TNF inhibitors in joints affected by rheumatoid arthritis, these inhibitors were insufficient to neutralize TNF activity (Cope et al, 1992). A randomized double blind study comparing administration of chimeric anti-TNF monoclonal antibodies to patients with rheumatoid arthritis resulted in an impressive clinical remission (Levine et al, 1994). Recently, it was demonstrated that incorporation of the sTNF-Rs into polymeric systems, such as ethylene-vinyl acetate copolymers or polylactic- glycolic acid and their subcutaneous injection, can provide systemic natural p55 sTNF-Rs at high concentrations, at a constant rate for prolonged periods (more than one month) (Eliaz et al, 1966). It is thus possible that sTNF-Rs will prove therapeutically effective in treating rheumatoid arthritis as well.

TNF, TNF-Rs and the Heart

Elevated concentrations of TNF and its soluble receptors have been detected in sera of patients with heart failure (Levine et al, 1990). TNF may contribute to the impaired myocardial contraction in this condition as it was shown to produce a significant depression of myocyte shortening (Cunnion, 1990). Furthermore, whole hearts perfused with serum from animals treated with TNF 18-22 hours earlier, exhibited significant impairment and decreased rate of relaxation compared to controls (DeMeules et al, 1992). Similar myocardial depressing effects

may possibly be inflicted by continuous exposure of the heart to TNF, circulating in heart failure patients (Levine et al, 1990). Neutralization of the cytokine with sTNF-Rs may be useful in managing heart failure.

5

Inhibition of TNF

Heparin has been reported to bind TNF (Lantz et al, 1991). However, the significance of this observation was never examined. The effects of heparin seem to be the exact opposite of the effects of TNF, as shown by Table I in Lantz et al.

10

Summary of the Invention

The present invention provides for the use of heparin, and/or a derivative thereof, in the preparation of a pharmaceutical composition for inhibiting the bioactivity of TNF.

15

The present invention also provides pharmaceutical compositions for inhibiting the bioactivity of TNF.

The invention provides further a kit for the simultaneous or sequential administration of such a composition, comprising the active ingredients together with a pharmaceutically acceptable carrier, and instructions for use.

20

Heparin and low molecular weight heparins have been found to inhibit the cytokine bioactivity of TNF, particularly when acting with another TNF binding protein. Heparin is a natural TNF binding protein, and probably cross-links TNF to its p55 TNF and p75 TNF-receptors. This inhibits the cytokine bioactivity of TNF by presumably interfering with trimerization of the TNF receptors. The inventors raise the above theory of action without being bound thereby. Thus, by administering heparin or a derivative thereof along with a soluble TNF receptor, the bioactivity of TNF is

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inhibited, and the disorders caused by excess TNF can be successfully treated. The heparin or derivative thereof can be administered simultaneously with the TNF receptor, either in separate compositions or in compositions containing both heparin or a derivative thereof and at least one soluble TNF receptor.

5

Brief Description of the Drawings

Figures 1A and 1B are graphs showing inhibition of TNF activity by heparin (Fig. 1A) and Clexane® (Fig. 1B).

10 Figure 2 is a graph showing the effect of pretreatment of cells with heparin on TNF cytotoxicity as a function of time the cells were exposed to heparin prior to TNF addition that heparin was added.

Figures 3A and 3B show the influence of supernatant removal on the cytotoxic effect of TNF.

Figure 4A shows the interactions of heparin with p55 sTNF-R and TNF.

15 Figure 4B shows the interactions of Clexane® with p55 sTNF-R and TNF.

Figure 5A shows the interaction of heparin with p75 sTNF-R and TNF.

Figure 5B shows the interactions of Clexane® with p75 sTNF-R and TNF.

20 Figure 6A shows the effect of heparin added at different time points after TNF application.

Figure 6B shows the effect of Clexane® added at different time points after TNF application.

25 Figure 7 illustrates interactions between TNF, soluble TNF-receptors, and heparin or low molecular weight heparin.

Figure 8 shows the equilibrium between TNF and its soluble receptors.

Detailed Description of the Invention

It has been discovered that heparin or low molecular weight heparin is able to enhance the effect of sTNF-Rs, apparently in a synergistic rather than merely an additive manner.

5 Heparin is a glycosaminoglycan, a highly sulfated mucopolysaccharide, consisting of a heterogeneous series of repeating disaccharide units composed of D-glucuronic or L-iduronic acids in a 1,4-glycosidic linkage to glucosamine. Each of the repeating units contains two sulfate esters and one N-sulfate group. Heparin is the strongest anionically charged organic acid substance ever isolated from a living
10 biological system. Heparin occurs in many different body tissues, but the lung, intestinal tract, liver, and mast cells are particularly rich in heparin. Heparin is a family of linear polymers that differ in chain length and molecular weight, and its precise complete composition is unknown.

 Commercially, heparin is extracted from animal tissues, most commonly
15 from bovine lungs and the intestinal mucosa of bovine, ovine, porcine, and caprine species. In any vial of therapeutically employed heparin, a wide range of molecular species, ranging from 2,000 to 25,000 daltons, are present. The potency of heparin is defined in units, where one unit is the amount of heparin that will prevent the coagulation of sheep plasma by the process of recalcification. Various extracts of
20 heparin may range in potency, i.e., 1 mg by weight may range in potency from 80-170 units. The World Health Organization maintains reference standards for heparin.

 Heparin exhibits its inhibitory effect on the blood coagulation cascade scheme by at least two different mechanisms. First, when heparin complexes with lysine residues of antithrombin III at high affinity in a 1:1 stoichiometric manner, the
25 serine protease inhibitory effect of antithrombin III is enhanced several fold. Second, because of its high polyanionic charge density, heparin is able to neutralize the effect of positively charged activated glycoprotein coagulant serine proteases. Heparin also

induces lipoprotein lipase and histaminase degradation of histamine, and has antiinflammatory properties as well.

Heparin has been widely used since the mid-1940s primarily for the prophylactic prevention and treatment of thrombotic diseases such as deep vein thrombosis, pulmonary emboli, and myocardial infarction. Another principal use of heparin is to prevent blood coagulation in extra-corporeal systems, thus making possible renal dialysis; cardiac bypass surgery; cardiac, pulmonary, hepatic, and renal transplantation; extra-corporeal pulmonary bypass oxygenation; and extra-corporeal circulatory membrane ultrafiltration. Low doses of low molecular weight heparin are used for the prophylactic prevention of intravascular thrombus formation. Heparin fragments, peptides, and synthetically prepared peptides have also been used.

In animal models, heparin has been shown to reduce the ability of autoimmune T lymphocytes to reach their target organ (Lider et al, 1990). Heparin has also been shown to suppress experimental autoimmune diseases in rats and to prolong the allograft survival in a model of skin transplantation in mice, when used in low doses of about 5 micrograms for mice and 20 micrograms for rats, injected once a day (Lider et al, 1989).

The mechanisms behind the observed effects of heparin are believed to involve inhibition of release by T lymphocytes of the enzyme(s) necessary for penetration of the vessel wall, primarily the enzyme heparanase that specifically attacks the glycosaminoglycan moiety of the sub-endothelial extracellular matrix that lines blood vessels (Naparstek et al, 1984). Expression of the heparanase enzyme is associated with the ability of autoimmune T lymphocytes to penetrate blood vessel walls and to attack the brain in the model disease experimental autoimmune encephalomyelitis.

Low molecular weight heparins, with an average molecular weight of 3000-6000, such as, for example, the low molecular weight heparins disclosed in

European Patent EP 0014184, are derived from heparin. Some low molecular weight heparins are commercially available under different trade names, such as Fragmin®, *cf.* U.S. Patent No. 4,303,651, Fraxiparin®, Fraxiparine®, U.S. Patents Nos. 4,486,420 and 4,692,435, Lovenox®, European Patent 40144, and Clexane®, U.S. Patent No. 3,948,917.

Low molecular weight heparins can be produced in several different ways: enrichment by fractionalization by ethanol and/or molecular sieving, e.g., gel filtration or membrane filtration of the low molecular weight heparin present in standard heparin and controlled chemical (by nitrous acid, *wbw*-elimination, or periodate oxidation) or enzymatic (by heparinase) depolymerization. The conditions for depolymerization can be carefully controlled to yield products of the desired molecular weights. Nitrous acid depolymerization is commonly used. Also, the benzylic ester of heparin can be depolymerized by *wbw*-elimination, which yields the same type of fragments as enzymatic depolymerization using heparinases. Low molecular weight heparin with low anticoagulant activity which retains the basic chemical structure of heparin can be prepared by depolymerization using periodate oxidation or by removing the antithrombin-binding fraction of low molecular weight heparin, prepared by other methods, using immobilized antithrombin for adsorption.

Fragmin® is a low molecular weight heparin with average molecular weight within the range of 4000-6000 dalton, produced by controlled nitrous acid depolymerization of sodium heparin from porcine intestinal mucosa. It is manufactured by Kabi Pharmacia, Sweden, under the name Fragmin® for use as an antithrombotic agent as saline solutions for injection in single dose syringes of 2500 IU/0.2 ml and 5000 IU/0.2 ml, corresponding to about 16 mg and 32 mg., respectively.

Fraxiparin® and Fraxiparine® are low molecular weight heparins with average molecular weight of approximately 4500 dalton, produced by fractionation or controlled nitrous acid depolymerization, respectively, of calcium heparin from

porcine intestinal mucosa. These low molecular weight heparins are manufactured by Sanofi (Choay Laboratories) for use as an antithrombotic agent in single doses comprising about 36 mg., corresponding to 3075 IU/0.3 ml water.

5 Lovenox®(Enoxaprain/e), a low molecular weight heparin fragment produced by depolymerization of sodium heparin from porcine intestinal mucosa using wbw-elimination, is manufactured by Pharmuka SF, France, and distributed by Rhone-Poulenc under the names Clexane® and Lovenox® for use as antithrombotic agents in single dose syringes comprising 20 mg/0.2 ml and 40mg/0.4 ml water.

10 Low molecular weight heparins, produced by fractionalization or controlled depolymerization of heparins, show improved antithrombotic performance but also different pharmacokinetic properties as compared to heparin. The half-life is doubled and the bioavailbailty is higher with respect to their anticoagulant effect following subcutaneous injection (Bratt et al, 1985; Bone et al, 1987).

15 The properties of the low molecular weight heparins described above are a common feature to all low molecular weight heparins, regardless of the manufacturing process, the structural differences (created by depolymerization or those dependent on variation in the heparin used as raw material) or the anticoagulant activity, provided the low molecular weight heparin used is capable of inhibiting TNF secretion *in vitro* by resting T cells and/or macrophages in response to activation by
20 contact with specific antigens, mitogens, disrupted extracellular matrix or its protein components, such as fibronectin or laminin.

To test the effectiveness of inhibition of activity of TNF (see Examples 1 and 2), WISH cells were seeded at a concentration of 30,000 cells per well in 100 µl medium. Sixteen hours later, when the cells had reached about 90% confluence, TNF,
25 receptors, heparin, or Clexane® were added to the wells. The final concentrations added to the respective wells are as follows:

TNF 0.5 ng/ml

Heparin, 1 unit/ml

Clexane®, 1 unit/ml

TBPI (p55 TNF-receptor), 5 ng/ml

5 TBPII (p75 TNF-receptor), 10 ng/ml

The different combinations were mixed in an Eppendorf tube reaching a final volume of 300 µl. Then, 50 µl were added to the respective well. If incubation was required, it was conducted in the Eppendorf tube for 30 minutes at 37°C, in a mixture or separately. After each of the different combinations were added, 50 µl
10 cycloheximide was added to each well. Sixteen hours later, the cell supernatants were discarded and neutral red dye was added for one hour at 37°C. The dye was extracted from surviving cells with a Sorensen's solution and the results were read in an ELISA reader.

The addition of one unit/ml of heparin to 0.5 ng/ml of TNF was found to
15 have about 25% of the bioactivity of TNF (i.e., a reduction of cytotoxicity from 49% to 37%), as shown in Fig. 1A. Clexane® had a very similar effect, as shown in Fig. 1B. Thus, both heparin and Clexane®, a low molecular weight heparin, inhibited the toxicity of TNF.

Heparin is believed to interfere with the cellular binding of TNF. As
20 detailed in Example 3, WISH cells were pretreated with heparin between 0 and 30 minutes prior to TNF addition. This pretreatment substantially inhibited TNF cytotoxicity, as shown in Fig. 2. The heparin inhibitory effect was expressed immediately (at time 0), suggesting that it is not due to a metabolic effect which induces cell resistance to TNF.

25 The possible explanations of this phenomenon are:

(1) Heparin may bind to TNF receptors (cell surface or soluble receptors), interfering with the binding of TNF to its receptors.

(2) Heparin does not affect TNF receptors. It may complex with TNF the moment it is applied, and/or may interfere with ligand binding to cell-associated receptors and prevent TNF from inducing receptor trimerization, which is a prerequisite for signal transduction.

5 As seen in Example 4, elimination of heparin or low molecular weight heparin from supernatant instantaneously eliminates their protective effect against TNF cytotoxicity. Thus, the presence of heparin or a low molecular weight heparin is required for inhibiting TNF activity. The polysaccharides do not induce a metabolic state of resistance in cells pretreated with these polysaccharides. There is no affinity
10 between heparin or low molecular weight heparin and cell membrane elements, such as TNF receptors, since simple mechanical washing practically removes it and abolished the TNF-inhibitory effects.

It may, therefore, be assumed that, since the "cellular effect" of heparin is removable, it is likely that the TNF- inhibitory effect of heparin is due to its
15 adherence to TNF, preventing its association with the cell membrane TNF-Rs or interfering with it.

As detailed in Example 5, examination of the binding of heparin or Clexane® to the soluble TNF receptors or to the complex TNF/TNF-R revealed the following, with reference to Figs. 4 and 5:

20 (1) Preincubation of TNF with heparin or Clexane® for 30 minutes, and their application to the WISH cells, further potentiated their TNF inhibitory effect compared to their applications without preincubation (compare Figs. 4A, 4B, 5A, 5B, columns 3 vs. 6). There appears to be an interference phenomenon. Following preincubation with heparin or low molecular weight heparin, more TNF is bound to
25 the polysaccharide which may interfere with TNF binding to its cell associated receptors. An alternative explanation is that heparin may promote dissociation of the active trimer into inactive monomers.

(2) While p55 sTNF-R or p75 sTNF-R alone could inhibit about 8-15% of the TNF bioactivity, the addition of either heparin or Clexane® to TNF and either receptor potentiated the inhibition by the receptors 3-4 times (60% inhibition). Compare Figs. 4 and 5, columns 2, 4 and 5.

5 Thus, heparin or low molecular weight heparin may augment the soluble TNF-R binding to TNF, potentiating three to four times the neutralizing effect of both p55 sTNF-R and p75 sTNF-R. It appears that the polysaccharide cross-links TNF to its receptors. Although the conclusion that heparin prevents TNF binding to its cell associated receptors and the conclusion that heparin augments this binding to the
10 soluble receptors seems contradictory and paradoxical, both conditions result in an inhibition of TNF bioactivity and may coexist.

(3) Simple preincubation of p55 sTNF-R with TNF, unlike its preincubation with p75 sTNF-R, resulted in superior TNF inhibition, as shown in Figs. 4A and 5A, and 4B and 5B, comparing columns 4 and 7. This may be related to
15 the "ligand passing" effect of p75 sTNF-R.

(4) Thirty-minute preincubation of TNF with p55 sTNF-R/p75 sTNF-R and heparin/Clexane® resulted in almost the same TNF inhibition observed when the three components were applied over cells without preincubation (*cf.* Figs. 4 and 5, comparing columns 5 to 8). From this, one can conclude that the interaction
20 among TNF, soluble receptor, and heparin is instantaneous, unlike the interaction of TNF-heparin, which is augmented with time, as shown above. This suggests that the natural tendency of TNF to bind instantaneously to its receptor may be followed by quick cross-linking of the complex formed by heparin/Clexane®.

(5) TNF was preincubated with heparin/Clexane® for thirty minutes
25 and just prior to their application to cells, the p55 sTNF-R or p75 sTNF-R, respectively, was added. The observed TNF cytotoxicity was higher compared to the simultaneous preincubation of the three components, as shown in Figs. 4 and 5,

comparing columns 8 to 9. One explanation is that, during the TNF+heparin/Clexane® incubation, the polysaccharide complexed with TNF, interfering with its binding to the soluble receptors upon their later addition. Since free TNF, heparin, and their complex were at equilibrium, elimination of free TNF by
5 addition of soluble receptors resulted in dissociation of TNF/polysaccharide complexes in order to regain the equilibrium, and free TNF had an equal chance to bind the soluble receptors of the cell receptors and activate them. Further support for heparin/Clexane®'s interference with TNF binding to its receptor was gained when comparing column 9 to column 5 in Figs. 4 and 5.

10 (6) Thirty-minute preincubation of p55 sTNF-R or p75 sTNF-R with either heparin/Clexane® and addition of TNF just before application to the cells, as shown in Figs. 4A and 5A, and 4B and 5B, resulted in TNF inhibition identical to that obtained if the three components were added simultaneously to cells (column 5) or after their joint preincubation for thirty minutes (column 8). From this it can be
15 concluded that the polysaccharide does not interfere with TNF-receptor binding to TNF, and has no affinity for the "bare" TNF receptor. However, heparin/Clexane® has a strong affinity for the TNF/TNF-receptor complex, which it avidly cross-links. Thus, since heparin/Clexane® has no affinity for the "bare" soluble TNF receptors, the ease of washing of the "cellular effect" of heparin/Clexane®, shown in Fig. 2, is
20 consistent with the conclusion that the heparin/Clexane® has no affinity for the "bare" cell associated receptors as well.

(7) The inhibition of TNF after its preincubation with its p55 sTNF-R and addition of heparin/Clexane® just before application to cells was better (Figs. 4A and 4B, column 11) than the inhibition obtained after TNF preincubation with p55
25 sTNF-R only, as shown by a comparison of Figs. 4A and 4B, columns 7 and 11. This suggests that heparin/Clexane® further facilitates the inhibition of TNF by p55 sTNF-R, probably by their cross-linking. It should be noted that following

heparin/Clexane® addition, the TNF inhibition by p75 sTNF-R was better (20-25%) than the inhibition of p55 sTNF-R (15%). This can be seen by comparing Figs. 4A and 4B, columns 7 and 11, with Figs. 5A and 5B, columns 7 and 11. Heparin/Clexane® potentiates TNF binding to its p55 sTNF-Rr and p75 sTNF-R. The greater TNF inhibition by p75 sTNF-R in the presence of the polysaccharide may be related to prevention of "ligand passing" by p75 sTNF-R when heparin/Clexane® cross-links it to TNF.

Heparin/Clexane® potentiates binding of TNF to its soluble receptors, thus augmenting their TNF inhibitory effect. However, one would expect that a similar enhanced binding to cell associated receptors, shown in Figs. 4 and 5, comparing column 2 to column 3, would result in enhanced TNF cytotoxicity. In practice, though, TNF's cytotoxicity was inhibited.

One theoretical explanation for this apparent paradox is that heparin/Clexane® promotes cross-linking of the bioactive TNF trimer to only one or two TNF receptors, thus interfering with the binding of the third receptor to it. Promoting such binding to soluble TNF receptors neutralizes TNF bioactivity. Obviously, potentiating TNF binding to only one or two cell surface receptors, while interfering with the final receptor aggregation into trimers, explains the above paradox, as signal transduction is best elicited upon aggregation of three cell surface receptors. Following cross-linking of one cell surface receptor to TNF by heparin/Clexane®, the polysaccharide may become interposed in a way that may prevent further cell surface TNF-receptor trimerization. On the other hand, if the TNF already induced receptor trimerization, heparin cannot cross-link this complex or interfere with its function. This may be the key to TNF inhibition by this polysaccharide.

In support of the above explanation, it was noted that there was a paradoxical effect in the experiments. In experiments where lower amounts of soluble

receptors were used, characterized by a minimal (less than 10% TNF inhibition), the potentiating effect of heparin/Clexane® was maximal (more than 60% inhibition). In experiments in which the receptors exerted a 50% inhibition, heparin/Clexane® had a marginal potentiating effect.

5 It appears that with very low amounts of soluble receptors, most TNF trimers can produce complexes with only one soluble receptor. These complexes are the probable target of heparin/Clexane® cross-linking, resulting in a remarkable TNF inhibition. If the amount of the soluble receptors is higher, two or more soluble receptors may bind to TNF, preventing their effective cross-linking of such complexes
10 by the polysaccharide. It remains to be demonstrated that these complexes are not stabilized by heparin/Clexane® to the same extent as are complexes of TNF and monomeric soluble receptor.

 Strong supporting evidence that the heparin/Clexane® cross-linking may be limited to complexes of TNF with one or, at most, two receptors comes from
15 comparison of columns 5 to 11 of Figs. 4 and 5. If an equilibrium was attained between TNF+p55 sTNF-R with formation of TNF complexes with one, two or three receptors, and then heparin/Clexane® was added (column 11), the TNF cytotoxicity was enhanced compared to simultaneous addition of the TNF, its receptors and heparin over cells (column 5). A possible explanation is that, in the latter situation,
20 TNF could initially bind one single receptor. This complex would be immediately cross-linked by heparin/Clexane®, preventing TNF from binding a second or third receptor.

 Additional supporting evidence can be found in the experiment to determine if heparin/Clexane® can inhibit the bioactivity of TNF if heparin/Clexane®
25 is added after TNF application. If heparin/Clexane® was applied at different time periods after TNF application, its inhibitory activity was still significant after 15 minutes, and marginally persistent after one hour. Surprisingly, in two experiments,

the inhibitory activity of heparin/Clexane® was about 7-15% at time 0, increased to 25% if applied 5-15 minutes after TNF, and decreased to 10% at one hour (Fig. 6).

One explanation for this paradoxical increase if heparin/Clexane® is added 5-15 minutes after TNF is that heparin/Clexane® binds avidly only one monomer-receptor to a trimer TNF. This binding interferes with further receptor trimerization, which is known to be followed by signal transduction.

One can visualize the binding of TNF to its receptors as a process during which, at the beginning, TNF is bound to one receptor, with time is bound to the second receptor, and with additional time to the third receptor. The fact that heparin/Clexane® can still inhibit TNF even one hour after its application suggests that it may interfere at this late stage with trimerization of the few last receptors. TNF molecules may be combined at this later stage with one receptor or two, and heparin/Clexane® interferes with the binding of the third, which would otherwise induce signal transduction.

This mechanism may explain the paradox that addition of heparin/Clexane® fifteen minutes following TNF application results in a better inhibition of TNF than when applied simultaneously. At time 0, heparin/Clexane® binds part of the TNF and may slightly interfere with its binding to the cell receptors, as noted above. If low concentrations of TNF are applied several minutes before heparin/Clexane®, TNF has the opportunity to bind to its receptors undisturbed, and application of the heparin now will cross-link optionally the TNF bound to receptor monomers.

It can thus be seen that treating a patient with heparin and/or a low molecular weight heparin can inhibit the bioactivity of TNF. The effect of heparin or derivatives thereof can be potentiated by administering p55 sTNF-R or p75 sTNF-R in combination with the heparin or derivative thereof. The heparin and soluble receptors can be administered simultaneously, or over approximately a 15-30 minute interval.

Alternatively, the heparin or derivative is administered, and approximately 15-60 minutes later p55 sTNF-R or p75 sTNF-R is administered.

While heparin *per se* can be administered, low molecular weight heparin, produced by fractionation or controlled depolymerization of heparins, has improved antithrombotic performance, as well as different pharmacokinetic properties, as compared to heparin. The half-life of the low molecular weight heparins is doubled. However, even though Bratt et al (1985) found that their bioavailability is higher with respect to their anticoagulant effect after subcutaneous injection, it should be noted from Figs. 4 and 5 that heparin and Clexane® interact with p55 sTNF-R, p75 sTNF-R, and TNF approximately the same. Thus, any low molecular weight heparin can be used in place of heparin for the purpose of the present invention.

The low molecular weight heparins that can preferably be used in the present invention include Clexane®, as described above, as well as Fragmin®, a low molecular weight heparin with average molecular weight within the range of 4000-6000 daltons, produced by controlled nitrous acid depolymerization of sodium heparin from porcine intestinal mucosa, manufactured by Kabi Pharmacia, Sweden. Also useful are Fraxiparin® and Fraxiparine®, low molecular weight heparins with average molecular weight of approximately 4500 daltons, produced by fractionation or controlled nitrous acid depolymerization, respectively, or calcium heparin from porcine intestinal mucous manufactured by Sanofi (Choay Laboratories).

For purposes of the present invention, heparin *per se* can be used, either alone or in combination with a low molecular weight heparin, regardless of the manufacturing process, the structural differences (created by depolymerization or those dependent on variation in the heparin used as raw material), or the anticoagulant activity. Alternatively, a low molecular weight heparin can be used alone.

The disorders that can be treated by inhibiting TNF activity according to the present invention are all disorders related to the presence of TNF and which

respond to inhibition of the bioactivity of TNF. Among these disorders are atherosclerosis and vasculitis and pathological processes related thereto; autoimmune diseases, such as rheumatoid arthritis, diabetes mellitus type I; allergies; graft rejection; acute and chronic inflammatory diseases, such as uveitis and bowel
5 inflammation; anorexia nervosa; hemorrhagic shock caused by septicemia; and opportunistic infections in AIDS-compromised individuals.

Heparin or a low molecular weight heparin, or mixtures thereof, is incorporated into pharmaceutical compositions, for example, as water solutions, possibly comprising sodium chloride, stabilizers, and other suitable non-active
10 ingredients. The preferred method of administration is by injection, subcutaneous or intravenous, but any other suitable mode of administration is encompassed by the invention.

The soluble TNF receptors, p55 sTNF-R and p75 sTNF-R, are likewise incorporated into pharmaceutical compositions, either alone or in combination with
15 heparin and derivatives thereof. The amounts of heparin or derivatives thereof administered depend upon the mode of administration. If a slow release preparation is administered, the amounts administered will be much lower than if administered intramuscularly or intravenously.

Pharmaceutical compositions for administration according to the present
20 invention can comprise at least one heparin or derivative thereof and at least one soluble TNF receptor, either separately or together, in a pharmaceutically acceptable form optionally combined with a pharmaceutically acceptable carrier. These compositions can be administered by any means that achieve their intended purposes. Amounts and regimens for the administration of a composition according to the
25 present invention can be determined readily by those with ordinary skill in the art of treating disorders related to excessive bioactivity of TNF.

For example, administration can be by parenteral, such as subcutaneous,

intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively or concurrently, administration can be by the oral route. The dosage administered depends upon the age, health and weight of the recipient, type of previous or concurrent treatment, if any, frequency of the treatment, and the nature of the effect desired.

Compositions within the scope of this invention include all composition comprising at least one heparin or derivative administered in combination with at least one soluble TNF receptor in an amount effective to achieve its intended purpose. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise about 0.1 to about 100 mg/kg body weight.

The following non-limiting examples will help to explain the present invention.

15 EXAMPLE 1: Inhibition of TNF Activity by Heparin

WISH cells were seeded at a concentration of 30,000 cells/well in 100 μ l medium. Sixteen hours later, either medium (control), heparin, TNF or a combination of TNF plus heparin was added. The final concentrations of each into the respective wells were as follows: TNF 0.5 ng/ml; heparin 1 unit/ml. After the various additions, cyclohexamide was added to each well (50 μ l) for a final concentration of 25 μ g/ml in the well. Sixteen hours later, the cell supernatants were discarded and neutral red dye was added for one hour. Following the one hour incubation, the dye was extracted with a Sorensen's solution, and the results were read in an ELISA reader. The results directly correlate with percent of cell killing. The results are shown in Fig. 1A. It can be seen that the addition of TNF inhibits about 25% of the bioactivity of TNF (reduction of cytotoxicity from 49% to 37%).

EXAMPLE 2: Inhibition of TNF Activity by Clexane®

The same procedure as in Example 1 was repeated except that Clexane® was substituted for heparin. The results are shown in Fig. 1B. It can be seen that substantially similar inhibitory effects are obtained.

5

EXAMPLE 3: Effect of Pre-Treatment with Heparin on TNF Cytotoxicity

The same procedure as in Example 1 was repeated except that the WISH cells were treated with heparin between 0-30 minutes before addition of the TNF. The results are shown in Fig. 2. It can be seen that substantially identical inhibitory effects are obtained throughout the timeline.

10

EXAMPLE 4: Effect of Heparin Removal by Washing

WISH cells were seeded at a concentration of 30,000 cells per well in 100 µl medium. Sixteen hours after seeding of the cells either heparin or Clexane® was added to respective wells, while medium alone was added to control wells. The heparin or Clexane® was added to a final concentration of 1 unit/ml. Six hours later, part of the wells pretreated with either heparin or Clexane® or with medium only were washed three times with fresh medium, and TNF was added to a final concentration of 0.5 ng/ml. The results are shown in Fig. 3A for heparin and Fig. 3B for Clexane®. It can be seen that heparin/Clexane® pretreatment reduced the TNF cytotoxicity by 33% as expected (from 60% to 40%) (compare columns 2 to 4 in Figs. 3A and 3B). However, simple washing of the cells treated with medium also resulted in a 25% reduction in the cell susceptibility to TNF. Cells pretreated with heparin or Clexane®, whose supernatants were washed before TNF addition, had an identical killing by TNF (compare columns 3 to 5 of Figs. 3A and 3B). Thus, elimination of heparin or Clexane® from the supernatants eliminates instantaneously their protective effect against TNF cytotoxicity.

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Removal of the supernatants, washing the cells and addition of new medium increases the cell resistance to TNF by 20%. It is possible that the removed supernatants contain a factor that facilitates TNF cytotoxicity and its elimination reduces the effect of TNF. Another possibility is that, following removal of the supernatants, there is rapid shedding of the cell surface TNF receptors, as previously found (Aderka, in press), which may induce some transient desensitization to TNF.

EXAMPLE 5: Effect of Heparin/Clexane® on TNF Receptors

WISH cells were seeded at a concentration of 30,000 cells/well in 100 μ l medium. Sixteen hours later, either medium alone (control), or sTNF-R (either p55 sTNF-R (Figs. 4A and 4B) or p75 sTNF-R (Figs. 5A and 5B)), and either heparin (Figs. 4A and 5A) or Clexane® (Figs. 4B and 5B) were added. The final concentrations of each in the respective wells were as follows: TNF 0.5 ng/ml; heparin 1 unit/ml; Clexane® 1 unit/ml; TBP-I (p55 TNF-receptor) 5 ng/ml; TBP-II (p75 TNF-receptor) 10 ng/ml.

Different combinations of the ingredients were mixed in an Eppendorf tube reaching a final volume of 300 μ l. 50 μ l were then added to each respective well. If an incubation was required, it was done in an Eppendorf tube as a mixture or separately for 30 minutes at 37°C.

After addition of the different combinations, cyclohexamide was added to each well (50 μ l) for a final concentration of 25 μ g/ml. Sixteen hours later, the cell supernatants were discarded and neutral red dye was added for one hour. Following the one hour incubation, the dye was extracted with a Sorensen's solution and the results were read in an ELISA reader.

The results are shown in Figs. 4A, 4B, 5A and 5B. In each of these figures, the first bar of the graph represents a control in which the Eppendorf tube included 300 μ l of medium only. In the second bar of each, the Eppendorf tube

included 2.1 μ l of TNF at a concentration of 2 ng/ml plus 20 μ l of medium.

In bar 3 of each figure, 1.8 units of either heparin or Clexane® (10 μ l) and 10 μ l of medium were added to the Eppendorf tube along with the addition of 280 μ l of TNF at a concentration of 2.1 ng/ml. The mixture was then immediately added
5 to the WISH cell wells.

With respect to the fourth bar, 6 ng p55 sTNF-R (10 μ l) were added to 10 μ l of medium and the same amount of TNF discussed above, immediately prior to addition of to the WISH cell wells. In Figs. 5A and 5B, double the amount of p75 sTNF-R (12ng/10 μ l) was used in place of p55 sTNF-R.

10 With respect bar 5, 6 ng of p55 sTNF-R or 12 μ g of p75 sTNF-R (10 μ l), 1.2 units of either heparin or Clexane® (10 μ l), and 280 μ l TNF at 2.1 ng/ml were added to the Eppendorf tube. The mixture was then immediately added to the WISH cell wells.

Bar 6 involves the same materials as discussed above for bar 3, except
15 that the TNF and either heparin or Clexane® were added simultaneously to the Eppendorf tube and then incubated together for 30 minutes prior to being added to the WISH cell wells. Similarly, bar 7 is the same as described above for bar 4, except that either the p55 sTNF-R or p75 sTNF-R was mixed with the TNF and incubated together for 30 minutes at 37°C before being added to the WISH cell wells. The
20 experiment of bar 8 is the same as that described above for bar 5, except that the TNF, either the p55 sTNF-R or the p75 sTNF-R, and either the heparin or Clexane® were mixed together and incubated for 30 minutes before being added to the WISH cell wells.

For bar 9, the TNF and either heparin or Clexane® were pre-incubated
25 together for 30 minutes before addition of either the p55 sTNF-R or p75 sTNF-R which were pre-incubated separately and then immediate addition to the WISH cell wells. For bar 10, either the p55 sTNF-R or the p75 sTNF-R and either the heparin or

Clexane® were pre-incubated together for 30 minutes before addition of the pre-incubated TNF and then immediate addition to the WISH cell wells. For bar 11, the TNF and either p55 sTNF-R or p75 sTNF-R were incubated together for 30 minutes before addition of either the pre-incubated heparin or Clexane® and then
5 immediate addition to the WISH cell wells.

As can be seen from a comparison of the various bars of Figs. 4A, 4B, 5A and 5B, pre-incubation of TNF with heparin/ Clexane® for 30 minutes and their application to the WISH cells further potentiated their TNF inhibitory effect compared to their application without pre-incubation (comparing bars 3 and 6 of each).
10 Furthermore, while p55 sTNF-R or p75 sTNF-R alone could inhibit about 8-15% of the TNF bioactivity, addition of either heparin or Clexane® to TNF and either receptor potentiated the inhibition by the receptors three to four times (60% inhibition) (comparing columns 2, 4 and 5 of the various figures).

Kits for the simultaneous or sequential administration of heparin and/or
15 a derivative thereof, and a soluble TNF receptor, are prepared in a conventional manner. Typically, such a kit will comprise, e.g. an ampoule of each of the active ingredients in a pharmaceutically acceptable carrier, a syringe, and written instructions for the simultaneous or sequential administration. For example, if simultaneous administration is desired, the contents of the ampoules may be mixed prior to injection
20 in either a suitable vessel, or in the syringe itself.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific
25 embodiments without undue experimentation and without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed

herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed functions may take a variety of alternative forms without departing from the invention. Thus the expressions "means to..." and "means for...", or any method step language, as may be found in the specification
5 above and/or in the claims below, followed by a functional statement, are intended to define and cover whatever structural, physical, chemical or electrical element or structure, or whatever method step, which may now or in the future exist which carries out the recited function, whether or not precisely equivalent to the embodiment or embodiments disclosed in the specification above, i.e., other means or steps for
10 carrying out the same function can be used; and it is intended that such expressions be given their broadest interpretation.

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WHAT IS CLAIMED IS:

1. The use of heparin. and/or a derivative thereof, in the preparation of a pharmaceutical composition for inhibiting the bioactivity of TNF.
2. The use according to claim 1, wherein at least one soluble TNF receptor (sTNF-R) is used together with heparin and/or a derivative thereof.
3. The use according to claim 2, wherein the soluble TNF receptor is selected from the group consisting of p55 sTNF-R and p75 sTNF-R.
4. The use according to claim 1, wherein the heparin derivative is a low molecular weight heparin.
5. The use according to claim 4, wherein the heparin derivative has a molecular weight between 2500 and 6500 daltons.
6. The use according to claim 2, wherein the heparin or derivative thereof is administered simultaneously with the sTNF-R.
7. The use according to claim 2, wherein the heparin or derivative thereof is administered within one hour after the s-TNF-R has been administered.
8. A composition for inhibiting the bioactivity of TNF, comprising an effective amount of heparin or a derivative thereof, or a mixture of heparin and a derivative thereof.
9. A composition according to claim 8, also comprising at least one sTNF-R.
10. A composition according to claim 8 or 9, further comprising a pharmaceutically acceptable carrier.
11. A composition according to claim 9, wherein the soluble TNF receptor is selected from the group consisting of p55 sTNF-R or p75 sTNF-R.
12. A composition according to claims 8-11, wherein the heparin derivative is a low molecular weight heparin.

13. A composition according to claim 12, wherein the heparin derivative has a molecular weight between 2500 and 6500 daltons.

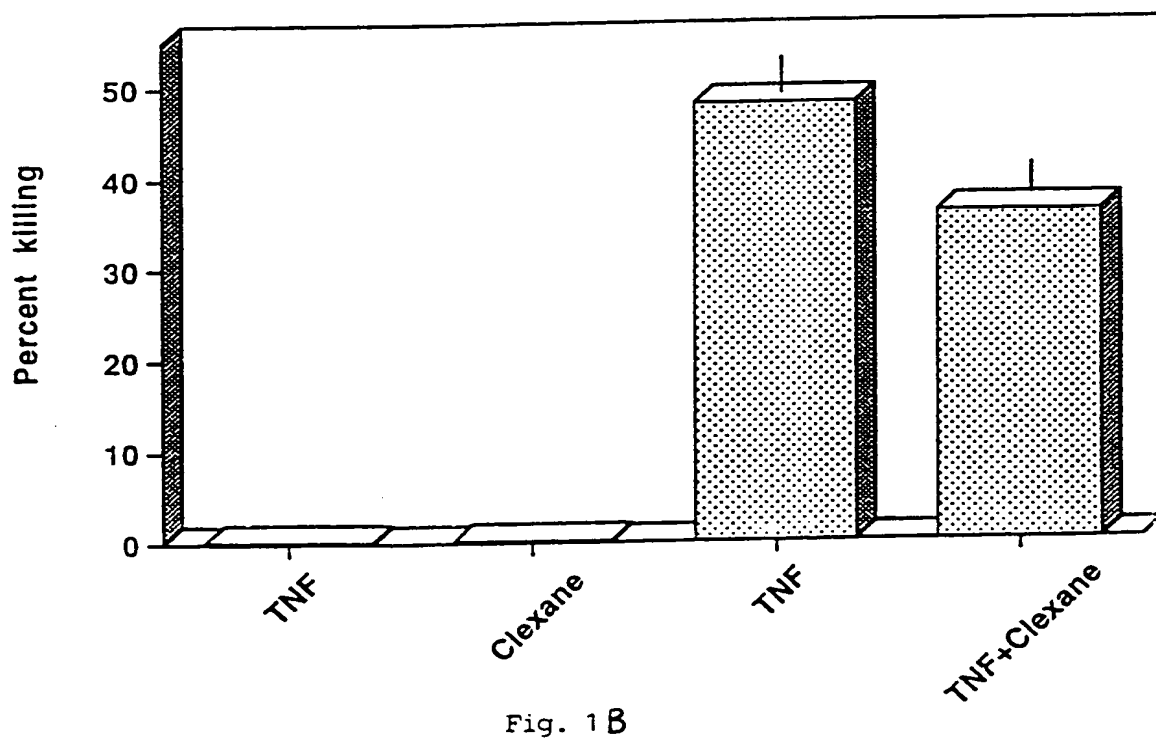
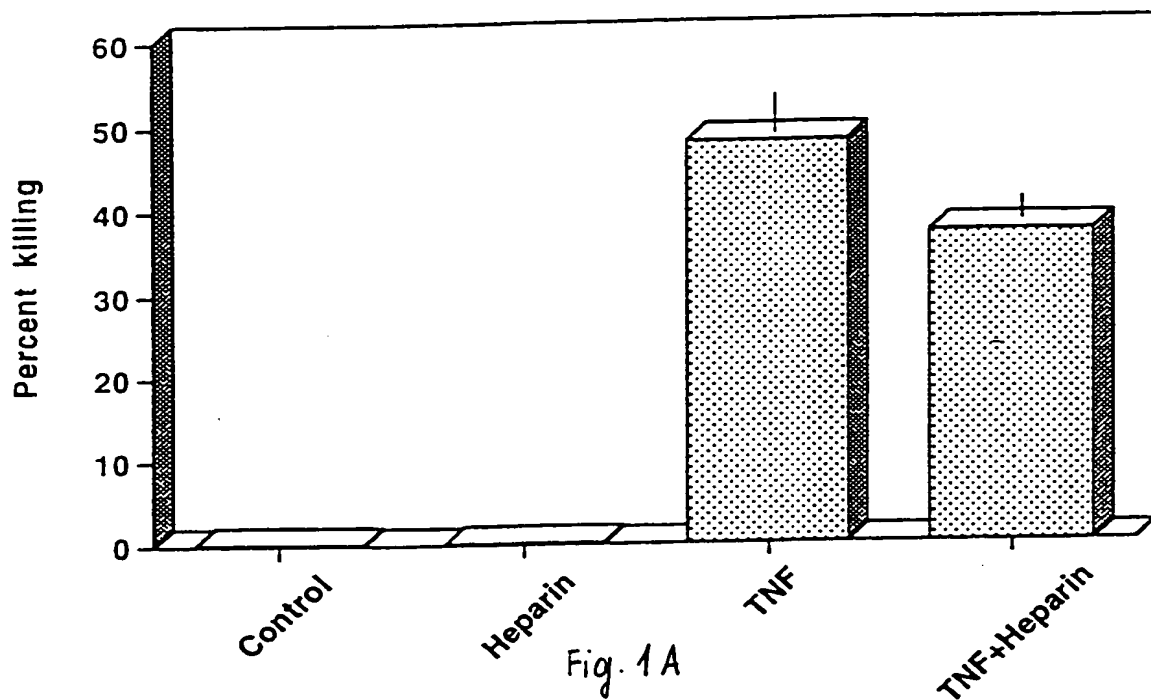
14. A kit for the simultaneous or sequential administration of a composition according to claims 8 to 13, comprising the active ingredients together with a pharmaceutically acceptable carrier, and instructions for use.

15. A composition comprising heparin and/or a derivative thereof and a sTNF-R.

16. A method for inhibiting activity of TNF in a subject comprising treating said subject with a pharmaceutical composition according to claims 8 to 13.

1/12

Inhibition of TNF activity by Heparin and Clexane



2/12

The effect of pretreatment with Heparin on TNF cytotoxicity

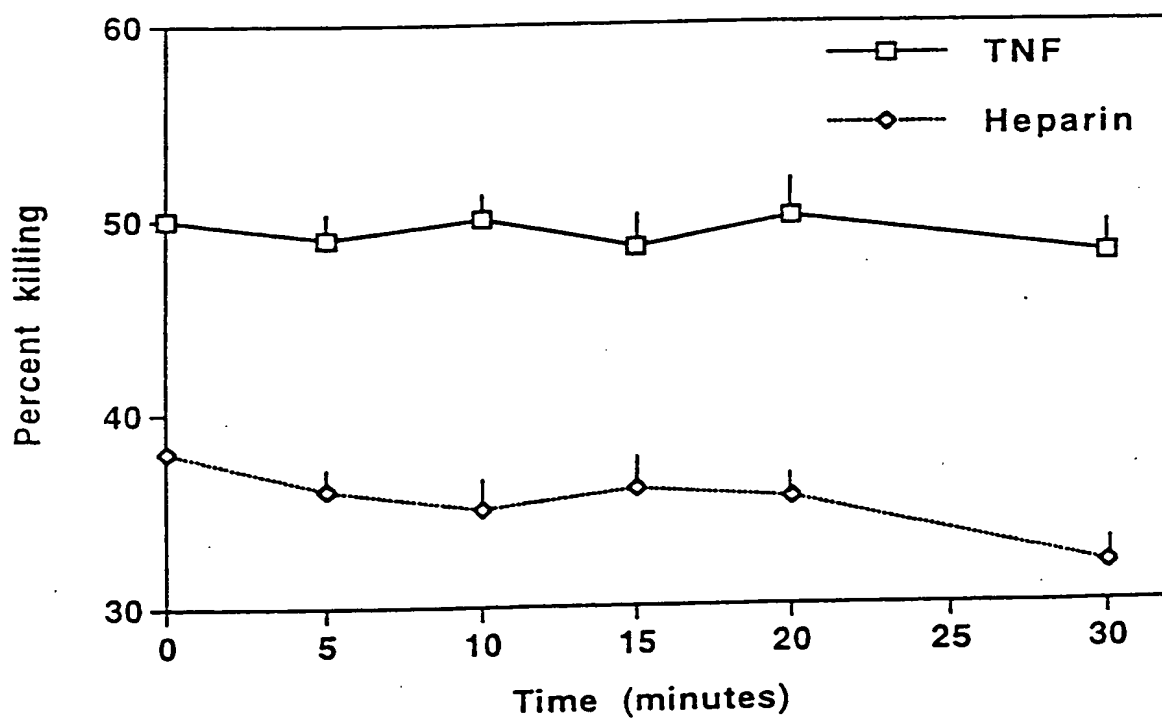


Fig. 2

3/12

The influence of supernatant removal on the cytotoxic effect of TNF

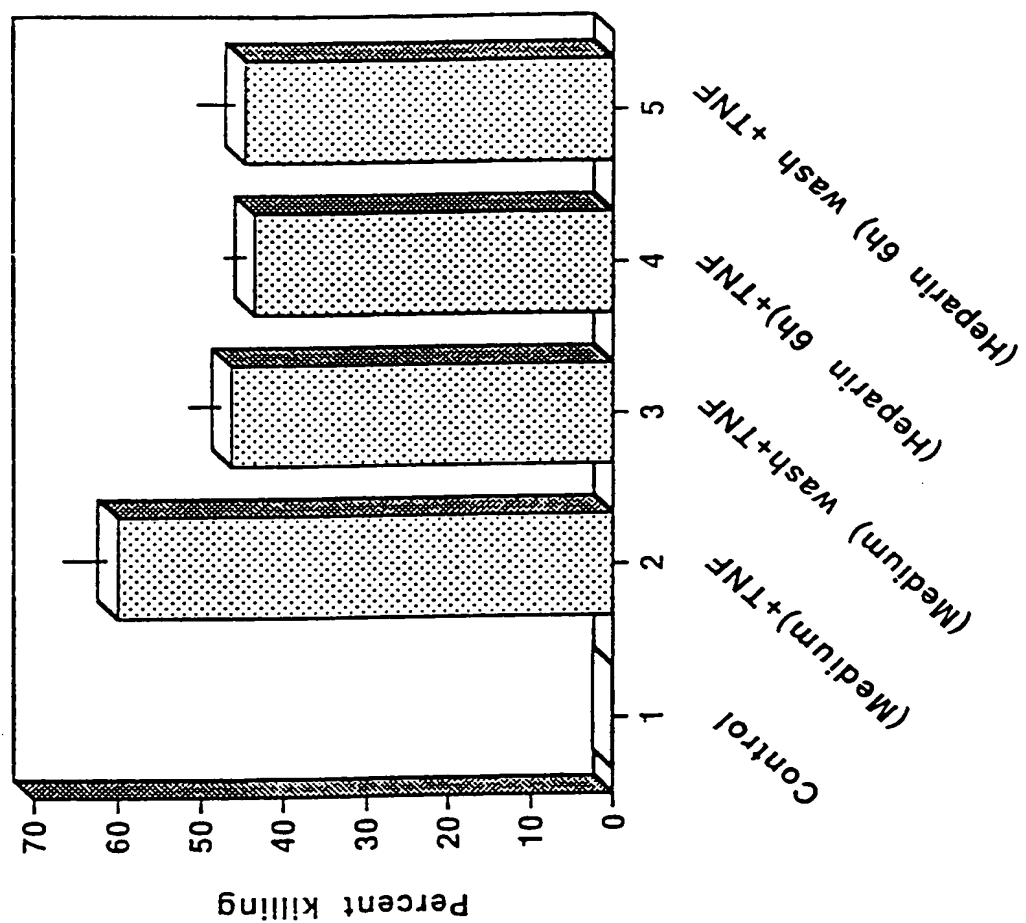


Fig. 3A

4/12

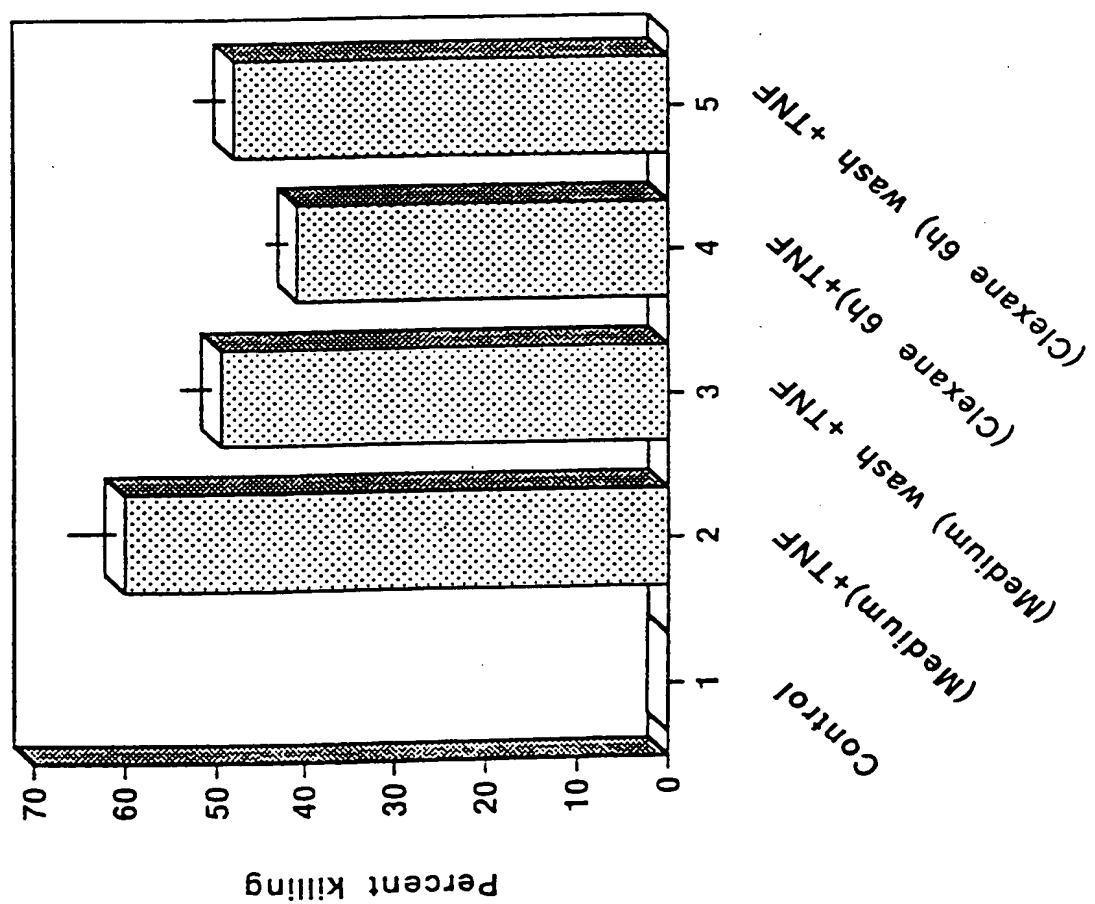


Fig. 3B

5/12

Heparin and Clexane: Interactions with p55 and TNF

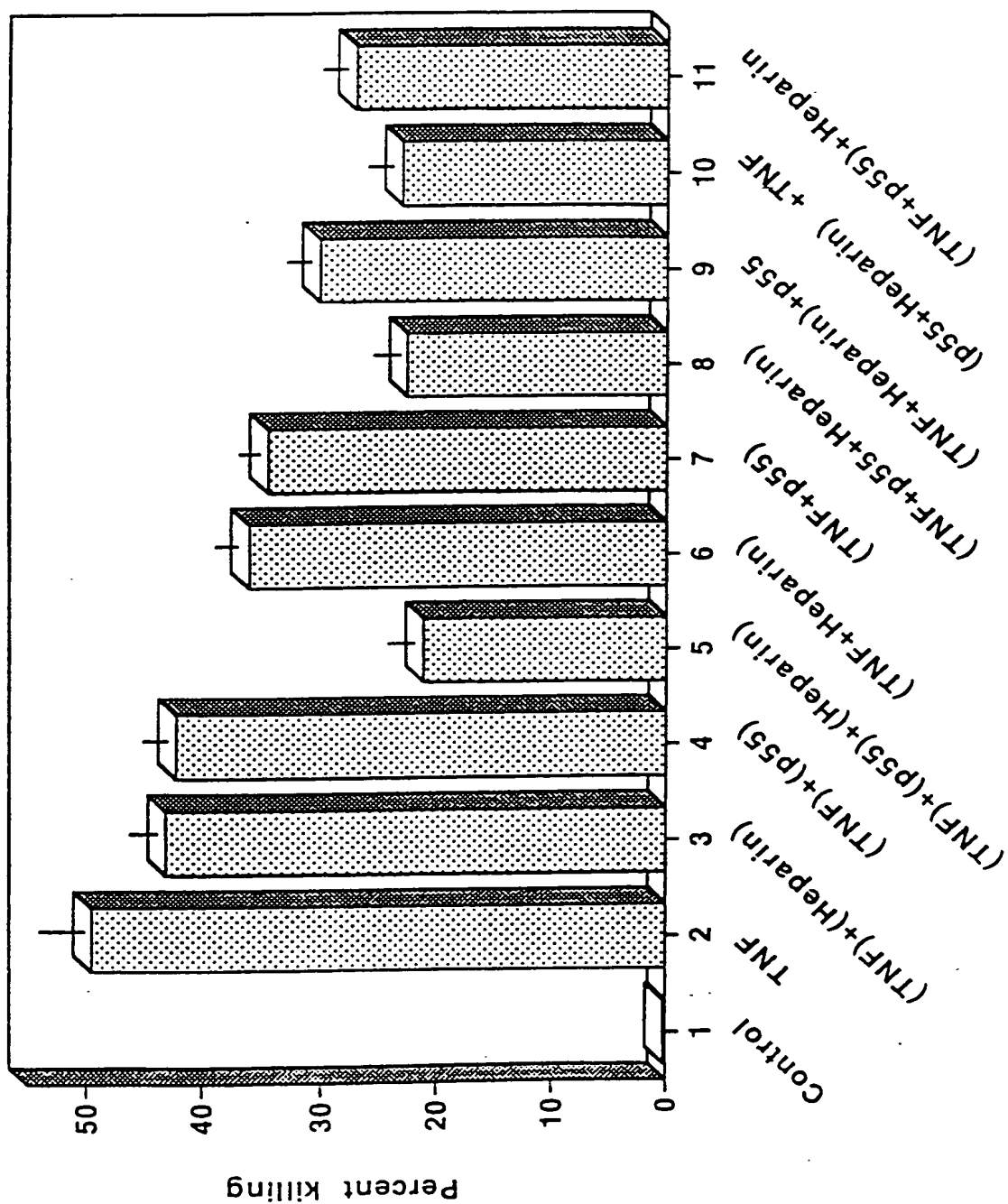


Fig. 4A

6/12

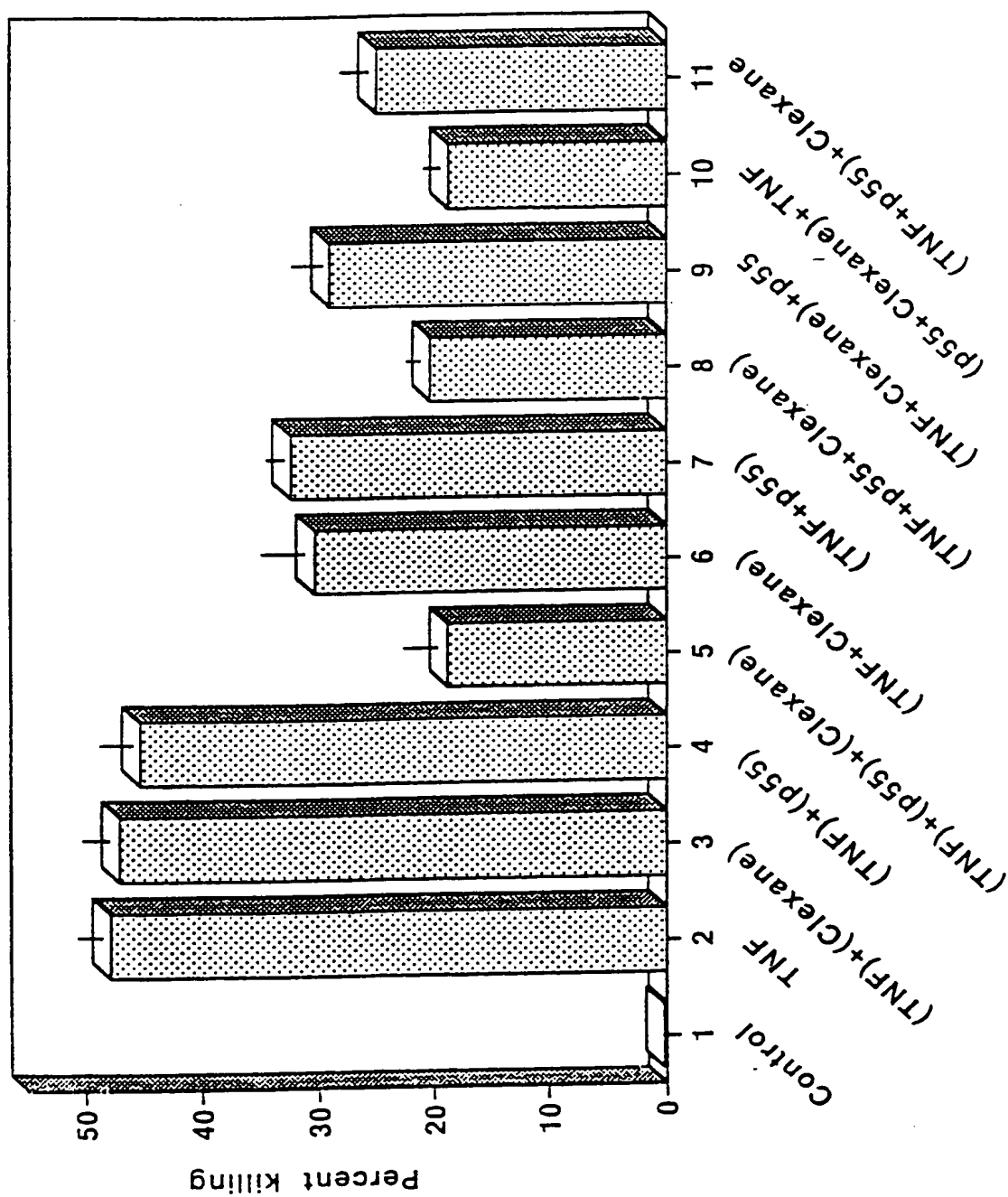


Fig. 4B

7/12

Heparin and Clexane: Interactions with p75 and TNF

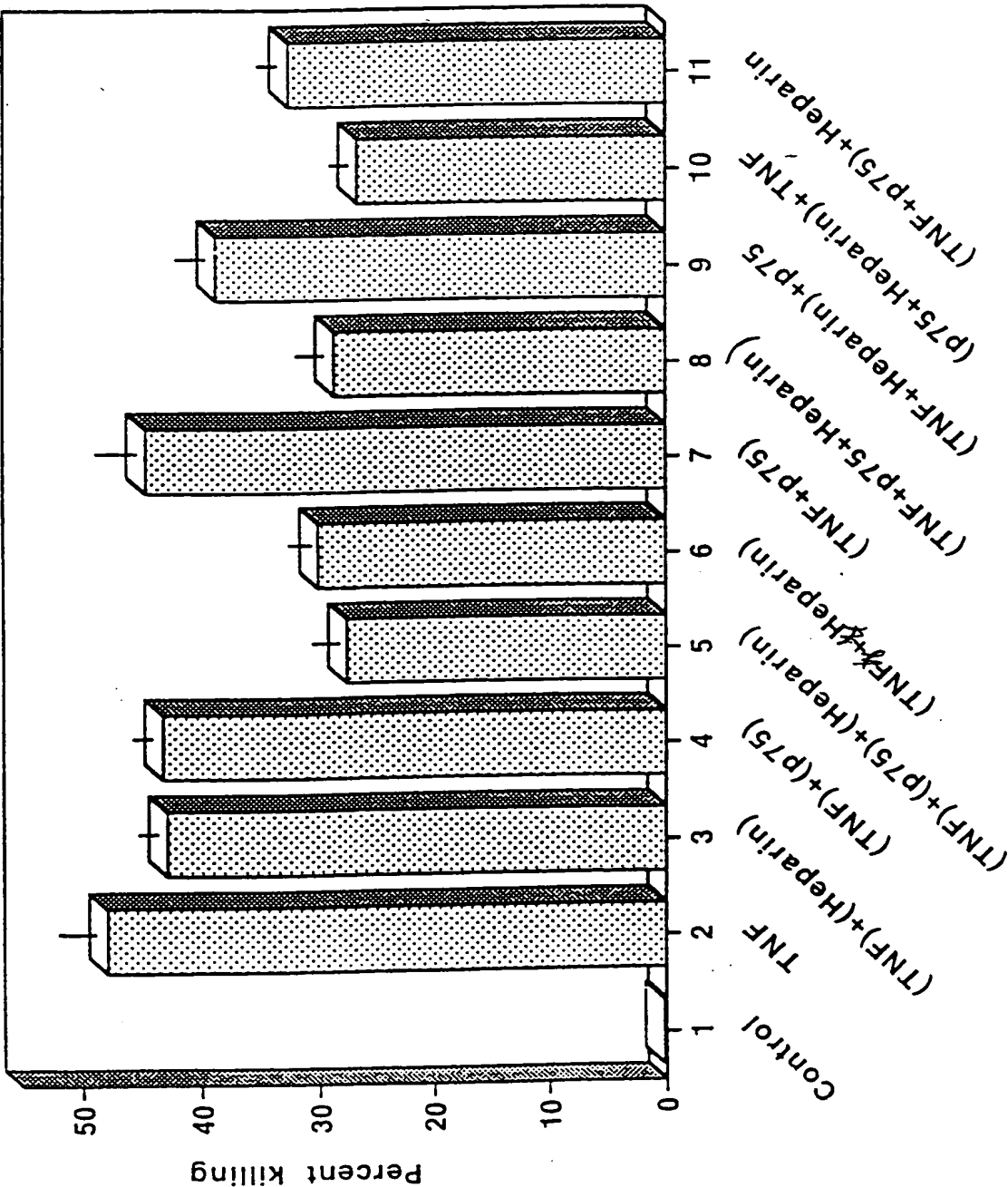


Fig. 5A

8/12

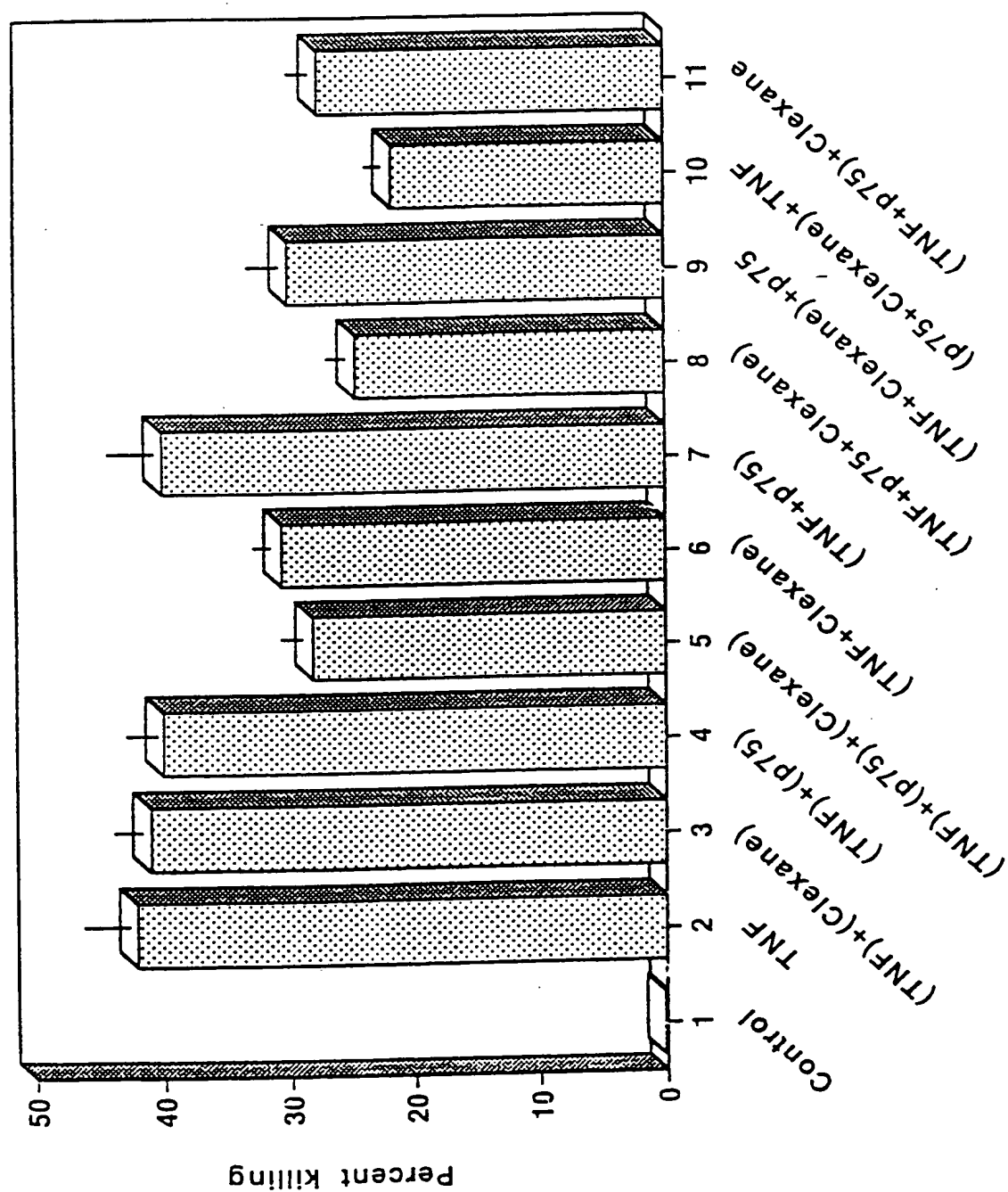


Fig. 5B

9/12

Wish experiment: Effect of Heparin or Clexane added at different time points after TNF application

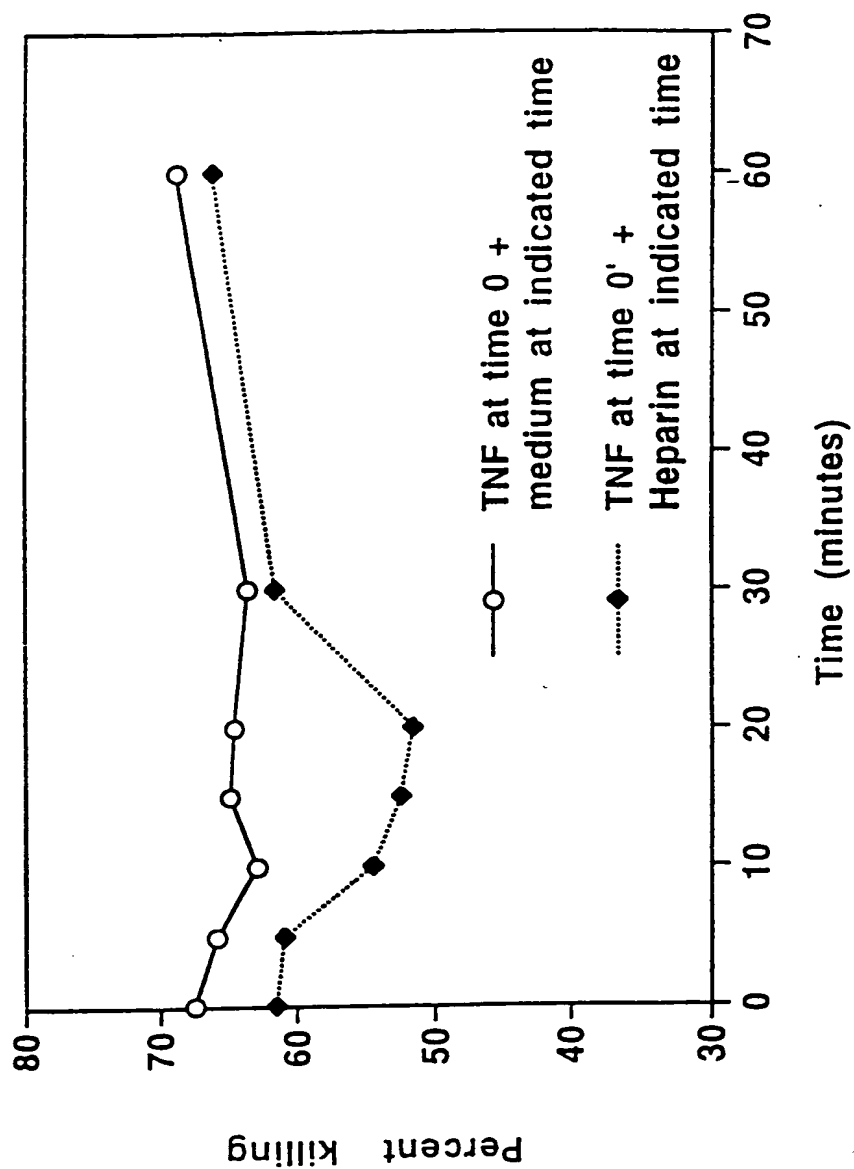


Fig. 6A

10/12

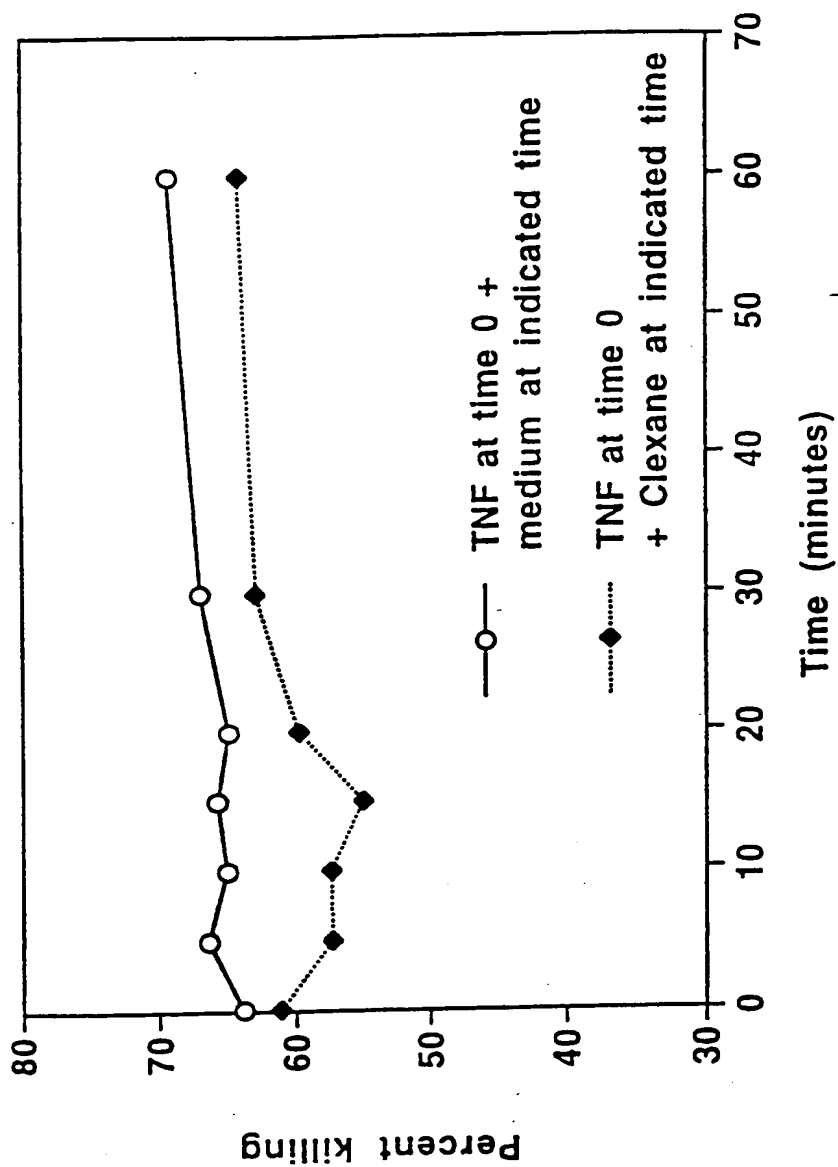
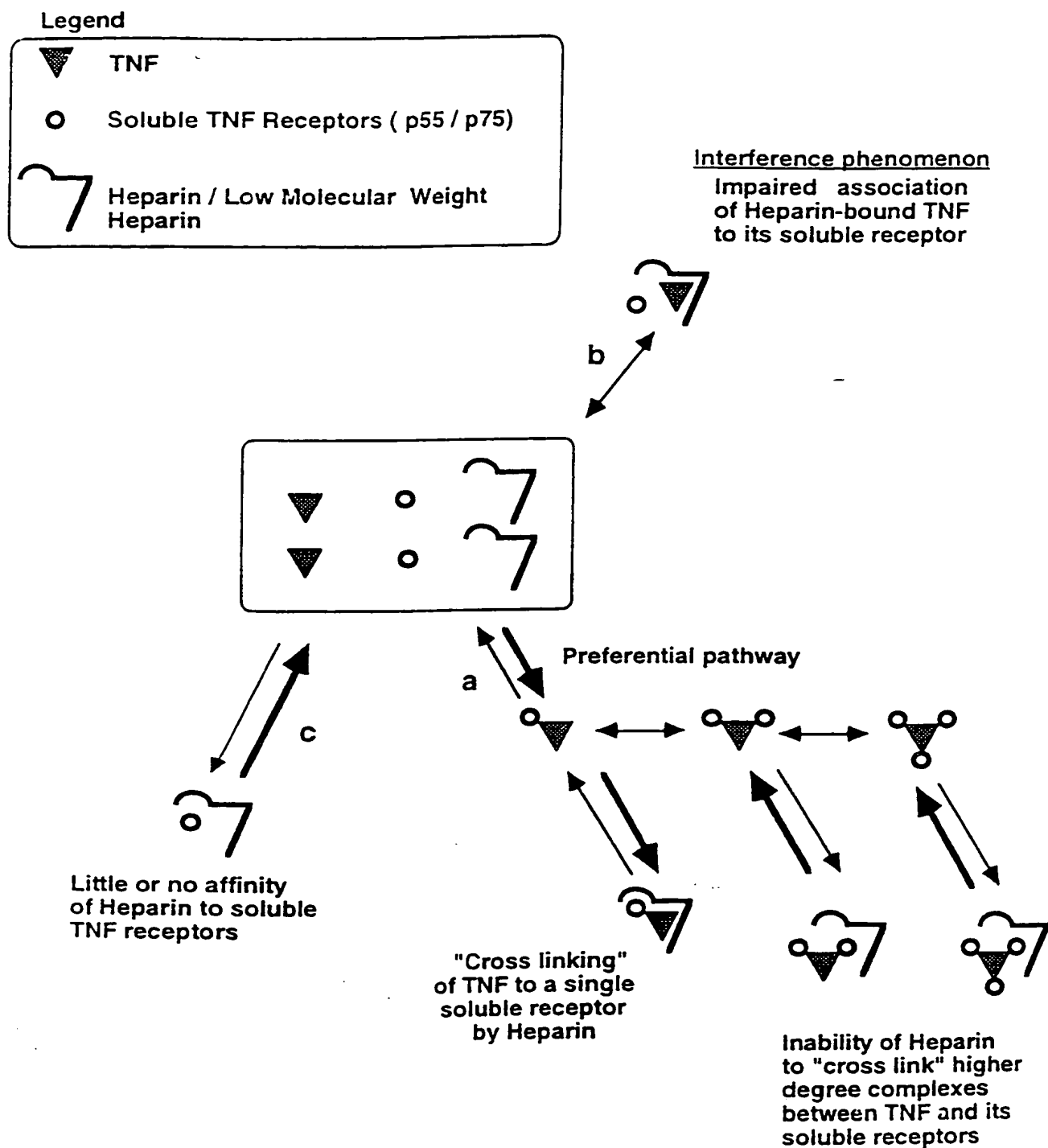


Fig. 6B

11/12

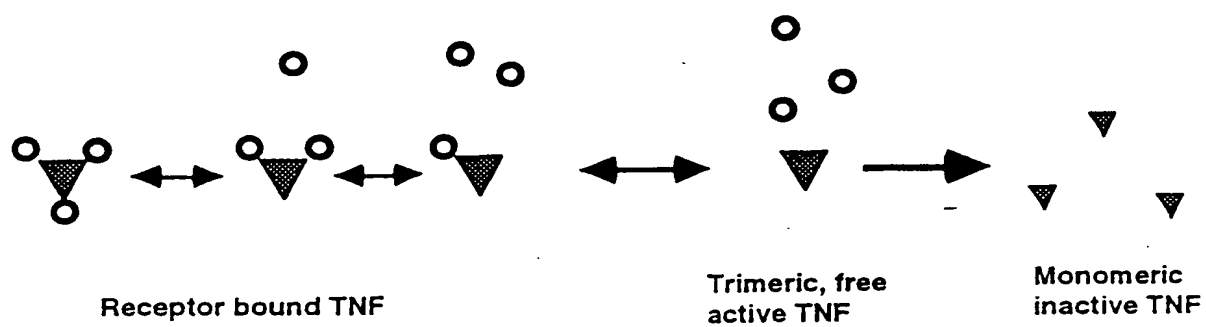


Interactions between TNF, soluble TNF-Receptors and Heparin or Low Molecular Weight Heparin

Fig. 7

12/12

The equilibrium between TNF and its soluble receptors



Legend

- ▼ TNF (trimer)
- ▼ TNF (monomer)
- Soluble TNF receptors (p55/p75)

Fig. 8

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(21) International Application Number: PCT/IL99/00709 (22) International Filing Date: 30 December 1999 (30.12.99) (30) Priority Data: 127851 30 December 1998 (30.12.98) IL (71) Applicant (for all designated States except US): APPLIED RESEARCH SYSTEMS ARS HOLDING N.V. [NL/NL]; Pietermaai 15, Curacao (AN). (72) Inventors; and (75) Inventors/Applicants (for US only): ADERKA, Dan [IL/IL]; Rechov Meridor 15, 69411 Tel Aviv (IL). ESHED (EN-GLENDER), Talma [IL/IL]; Rechov Geva 6, 53316 Givatayim (IL). (74) Agent: EINAV, Henry; Interpharm Laboratories Ltd., Science-based Industrial Park, Kiryat Weizmann, 76110 Ness-Ziona (IL).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> - (88) Date of publication of the international search report: 21 September 2000 (21.09.00)
(54) Title: INHIBITION OF TNF ACTIVITY (57) Abstract The bioactivity of TNF is inhibited by administering heparin or a derivative thereof along with a soluble TNF receptor. The heparin or derivative thereof can be administered simultaneously with the soluble TNF receptor, either in separate compositions or in compositions containing both heparin or a derivative thereof and at least one soluble TNF receptor. The heparin or derivative may also be administered without the soluble TNF receptor and still effect some amount of inhibition of TNF bioactivity.		

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INTERNATIONAL SEARCH REPORT

Int. lional Application No

PCT/IL 99/00709

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/727 A61K38/17 A61P37/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, BIOSIS, MEDLINE, CHEM ABS Data, SCISEARCH, CANCERLIT, AIDSLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 19249 A (COHEN IRUN R ;LIDER OFER (IL); YEDA RES & DEV (IL); HERSHKOVIZ RAM) 12 November 1992 (1992-11-12) abstract page 6, line 32 -page 7, line 13 page 8, line 7 -page 9, line 28 page 12, line 30 -page 13, line 7 claims 1,2,7-13,18,20,22-24 -- -/-	1,4,5,8, 10, 12-14,16

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

27 June 2000

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INTERNATIONAL SEARCH REPORT

Int lional Application No

PCT/IL 99/00709

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>BARAM D; RASHKOVSKY M; HERSHKOVIZ R; DRUCKER I; RESHEF T; BEN-SHITRIT S; MEKORI Y A: " Inhibitory effects of low molecular weight heparin on mediator release by mast cells: Preferential inhibition of cytokine production and mast cell-dependent cutaneous inflammation." CLINICAL AND EXPERIMENTAL IMMUNOLOGY, vol. 110, no. 3, December 1997 (1997-12), pages 485-491, XP000913798 abstract page 485, column 1, paragraph 1 page 485, column 2, paragraph 2 -page 486, column 1, paragraph 1 page 487, column 1, paragraph 2 -column 2, paragraph 1 page 490, column 1, paragraph 3</p>	1,4,5,8, 10, 12-14,16
X	<p>IMIELA JACEK; NOSARZEWSKI JERZY; GORSKI ANDRZEJ: "Oral heparin in the treatment of rheumatoid arthritis." ARCHIVUM IMMUNOLOGIAE ET THERAPIAE EXPERIMENTALIS, vol. 43, no. 5-6, 1995, pages 313-315, XP000913796 abstract page 313, column 1, paragraph 1 - paragraph 2 page 314, column 2, paragraph 2</p>	1,8,16
X	<p>KEPROS JOHN P; WANG PING; MORRISON MARY H; CHAUDRY IRSHAD H: "Mechanism of the beneficial effects of a novel nonanticoagulant heparin (GM1892) following hemorrhagic shock: Down-regulation of proinflammatory cytokine (TNF and IL-6) production." SURGICAL FORUM, vol. 46, 1995, pages 87-89, XP000913789 the whole document</p>	1,8,16
X	<p>BRAZIER F; YZET T; DESSAINT C; DUCHMANN J C; PRIN L; DUPAS J L: "Effect of heparin on interleukin-6 and tumor necrosis factor alpha serum levels in inflammatory bowel diseases." GASTROENTEROLOGY, vol. 110, no. 4 SUPPL., 1996, page A871 XP000913780 the whole document</p>	1,8,16

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Int. Patent Application No.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>OLSSON I; GATANAGA T; GULLBERG U; LANTZ M; GRANGER G A: "Tumour necrosis factor (TNF) binding proteins (soluble TNF receptor forms) with possible roles in inflammation and malignancy." EUROPEAN CYTOKINE NETWORK , vol. 4, no. 3, 1993, pages 169-180, XP000914174 abstract page 170, column 1, paragraph 1 page 171, column 2, paragraph 1 page 173, column 2, paragraph 4 -page 174, column 2, paragraph 2 figure 3 page 175, column 1, paragraph 2 page 175, column 2, paragraph 3 page 176, column 1, paragraph 2 page 177, column 2, paragraph 2</p>	<p>1-3,6, 8-11,15, 16</p>
A	<p>WO 95 03827 A (BAKER DAVID ;FELDMANN MARC (GB); KENNEDY INST OF RHEUMATOLOGY (GB)) 9 February 1995 (1995-02-09) abstract page 2, line 1 - line 27 page 4, line 28 - line 30 page 11, line 8 - line 17 examples 8,9 claims 13-24 figures 11,12</p>	<p>2,3,6,7, 9-11,14, 15</p>
A	<p>WO 94 06476 A (IMMUNEX CORP) 31 March 1994 (1994-03-31) abstract page 1, line 15 - line 17 page 2, line 3 - line 15 page 3, line 12 - line 25 page 5, line 12 - line 13 page 6, line 17 - line 18 example 4 page 23, line 29 - line 36 claims 1-5,8,9</p>	<p>2,3,6,7, 9-11,14, 15</p>

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/IL 99/00709

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>ADERKA DAN: "The potential biological and clinical significance of the soluble tumor necrosis factor receptors." CYTOKINE & GROWTH FACTOR REVIEWS, vol. 7, no. 3, 1996, pages 231-240, XP000913779 abstract page 231, column 1, paragraph 1 page 231, column 2, paragraph 4 -page 232, column 1, paragraph 1 page 232, column 2, paragraph 5 -page 233, column 1, paragraph 1 page 234, column 2, paragraph 2 -page 235, column 2, paragraph 2 page 236, column 1, paragraph 1 - paragraph 2 page 236, column 2, paragraph 3 tables 1,2</p> <p>---</p>	2,3,6,7, 9-11,14, 15
A	<p>EP 0 512 528 A (YEDA RES & DEV) 11 November 1992 (1992-11-11)</p> <p>abstract page 2, line 1 - line 17 page 2, line 36 - line 55 page 3, line 3 - line 5 examples 1,2 claims</p> <p>---</p>	2,3,6,7, 9-11,14, 15
P, X	<p>WO 99 09051 A (MEIJER HANS ;WEHLING PETER (DE); REINECKE JULIO (DE); ORTHOGEN GEN) 25 February 1999 (1999-02-25) page 1, paragraph 1 page 3, paragraph 1 page 5, paragraph 2 page 7, paragraph 2 claims 1,4;8</p> <p>-----</p>	8-11,14, 15

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-16 relate to a composition, its use and a method of treatment defined by reference to the parameter "inhibiting the bioactivity of TNF". Moreover, claims 1,2,6-11,14-16 relate to a composition, its use and a method of treatment defined by reference to the parameter "a derivative thereof" and claims 2,6,7,9-10,12-16 relate to a composition, its use and a method of treatment defined by reference to the parameter "a soluble TNF receptor". The use of these parameters in the present context is considered to lead to a lack of clarity within the meaning of Article 6 PCT. It is impossible to compare the parameters the applicant has chosen to employ with what is set out in the prior art. The lack of clarity is such as to render a meaningful complete search impossible. Consequently, the search has been restricted to the compounds for which pharmacological data are provided, namely heparin and low molecular weight heparin, p55 TNF-R and p75 TNF-R, and the disorders in which TNF is implicated and which are described on p.20, line 26 - p.21, line 6, with due regard to the general idea underlying the application.

Claims searched partially: 1-16.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IL 99/00709

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